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Abstract: It has been hypothesized that amphipathic peptides might bind to membranes prior to activating their cognate receptors, but this has proven difficult to test. The peptide hormone PYY3-36 is believed to perform its appetite-suppressing actions through binding to hypothalamic Y2 receptors. It has been proposed that PYY3-36 via its amphipathic a-helix binds to the plasma membrane prior to receptor docking. Here, our aim was to study the implication of this hypothesis using new analogs of PYY3-36. We first studied membrane binding of PYY3-36. Next, we designed a series of PYY3-36 analogs to increase membrane-binding affinity by substituting the N-terminal segment with a de novo designed a-helical, amphipathic sequence. These 2-helix variants of PYY3-36 were assembled by solid-phase peptide synthesis. Pharmacological studies demonstrated that even though the native peptide sequence was radically changed, highly active Y2 receptor agonists were generated. A potent analog, with a Kd of 4 nM for membranes, was structurally characterized by NMR in the membrane-bound state, which clearly showed that it formed the expected 2-helix. The topology of the peptide–micelle association was studied by paramagnetic relaxation enhancement using a spin label, which confirmed that the hydrophobic residues bound to the membrane. Our studies further support the hypothesis that PYY3-36 associates with the membrane and indicate that this can be used in the design of novel molecules with high receptor binding potency. These observations are likely to be generally important for peptide hormones and biopharmaceutical drugs derived from them. This new 2-helix variant of PYY3-36 will be useful as a tool compound for studying peptide–membrane interactions.

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Improving membrane binding as a design strategy for amphipathic peptide hormones: 2-helix variants of PYY3-36

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PYY3-36, Y2 receptor agonist, membrane binding, NMR, gut hormone, obesity

Abstract

It has been hypothesized that amphipathic peptides might bind to membranes prior to activating their cognate receptors, but this has proven difficult to test. The peptide hormone PYY3-36 is believed to perform its appetite suppressing actions through binding to hypothalamic Y2 receptors. It has been proposed that PYY3-36 via its amphipathic α-helix binds to the plasma membrane prior to receptor docking. Here our aim was to study the implication of this hypothesis using new analogs of PYY3-36. We first studied membrane binding of PYY3-36. Next, we designed a series of PYY3-36 analogs to increase membrane-binding affinity by substituting the *N*-terminal segment with a *de novo* designed α-helical, amphipathic sequence. These 2-helix variants of PYY3-36 were assembled by solid-phase peptide synthesis. Pharmacological studies demonstrated that even though the native peptide sequence was radically changed, highly active Y2 receptor agonists were generated. A potent analog, with a Kd of 4 nM for membranes, was structurally characterized by NMR in the membrane-bound state, which clearly showed that it formed the expected 2-helix. The topology of the peptide-micelle association was studied by paramagnetic relaxation enhancement using a spin-label, which confirmed that the hydrophobic residues bound to the membrane. Our studies further supports the hypothesis that PYY3-36 associates with the membrane, and indicate that this can be used in the design of novel molecules with high receptor binding potency. These observations are likely to be generally important for peptide hormones and biopharmaceutical drugs derived from them. This new 2-helix variant of PYY3-36 will be useful as a tool compound for studying peptide-membrane interactions.

Introduction

Neuropeptide Y (NPY), pancreatic polypeptide (PP) and peptide YY (PYY) belong to the NPY family of peptides. These peptide hormones are ligands of the so-called Y receptors (Y1, Y2, Y4, Y5),[1] that are involved in a variety of physiological functions, including central (CNS) and peripheral control of blood pressure, heart rate, food intake, gut motility, gastric, and gut secretions.[2-3] Recent data have indicated a role for peripherally circulating PYY3-36 in appetite regulation, presumably via interaction with hypothalamic Y2 receptors,[3-5] which has led to considerable efforts into developing novel PYY3-36 peptide analogs for the treatment of obesity both by us[6-9] and others[10-14].

PYY is co-secreted with the glucagon-like peptides 1 and 2 as well as oxyntomodulin from the endocrine L-cells lining the gut following the ingestion of a meal. Full-length PYY1-36 is a high-affinity Y1, Y2 and Y5 receptor ligand, but *N*-terminal truncation of PYY1-36 by dipeptidyl peptidase IV (DPP-IV)[15] generates PYY3-36 which is more selective for the Y2 (and Y5) receptors and believed to be the natural ligand of the Y2 receptor.[4-5] Although scientific efforts over the past three decades have greatly enhanced our understanding of the Y receptor pharmacology, there is still only a limited understanding of the molecular and biophysical mechanisms underlying the selectivity shifts of PYY and NPY towards the Y2 receptor upon *N*-terminal truncation. Kaiser and Kezdy, and then Schwyzer have suggested that amphipathic peptides might bind to membranes before binding to their membrane-bound receptors and that the membrane binding induces a conformation in the peptides suitable for receptor binding (the membrane compartment model).[16-19] According to the membrane compartment model amphipathic peptides that bind to GPCRs are bifunctional, where one part of the peptide (*the address*) directs the peptide towards the membrane, while another part (*the message*) binds to and activates the receptor.[19]

Recently, the membrane compartment model has been applied to study the NPY family of peptides and has shed new light on the peptide receptor binding mechanism. In peptides from the NPY family, the amphipathic α-helix was proposed to constitute the 'address', while the *C*-terminal pentapeptide would form direct contacts with the receptor.[20] Comparison of structures of PP, NPY and PYY in the absence and presence of a membrane model indicate that prior to receptor binding the ligand associates with the membrane.[20-22] This is based on NMR studies which have shown that full-length PYY1-36 in solution consists of a *C*-terminal receptor-recognizing pentapeptide that is attached to an amphipathic α-helix and followed by a *N*-terminal polyproline helix, and that hydrophobic contacts are formed between the α-helix and the *N*-terminal polyproline type.[21] Upon membrane association this hydrophobic contact is disrupted, and the hydrophobic face of the α-helix binds to the membrane surface and the *N*-terminal segment is released to freely diffuse in solution.[21] PYY3-36 truncated to PYY22-36 has very poor

receptor binding,[13] which could indicate that the *N*-terminus either assists in binding to the receptor or binds to the membrane.

Here, we first studied the membrane-binding properties of PYY3-36 using fluorescence and CD methods. Second, we designed potent Y2 receptor agonists with improved membrane surface binding. This was achieved by replacing the whole *N*-terminus of PYY with a de novo designed amphipathic α-helix (Figure 1), rather than by attaching a lipid which will insert into the membrane. The native α-helix was maintained as it likely plays a role in enabling binding of the *C*-terminal pentapeptide to the receptor. We speculated that the introduction of an additional amphipathic α-helix would provide peptides with a higher membrane affinity, due to an increased number of hydrophobic contacts with the outer leaflet of the membrane, which could result in an increased accumulation in the vicinity of the Y receptors. As the native *N*-terminus appears to be important for Y1 receptor affinity, we expected that this strategy could improve the Y2/Y1 receptor subtype selectivity. The peptides were assembled by solid-phase peptide synthesis, using a Fmoc protocol. Indeed, our work demonstrates that the additional amphipathic α-helix generally led to potent Y2 receptor agonists. In addition, sustained or improved selectivity for the Y2 subtype was observed. Moreover, as intended by the novel design of the 2-helix PYY analogs, they associated with the membrane with much higher affinity.

Methods

Peptide synthesis

The peptides were synthesized on Rink Amide TentaGel resin using the Fmoc/*t*Bu strategy by automated solid-phase peptide synthesis on a Biotage/MultiSynTech SyroII or Syro *Wave*™[23]. The N^{α}-Fmoc-protected amino acids (4 eq.) were coupled using HBTU (3.8 eq.) as coupling reagent, and HOBt (3.6 eq.) and HOAt (0.4 eq.) as additive, DIEA as base (7.8 eq.), in DMF. Coupling times were generally 2 × 2 h at room temperature or 2 × 10 min at 75 ºC by microwave-assisted SPPS[24]. N^{α}-deprotection was performed using piperidine in DMF. The peptides were side-chain deprotected and simultaneously cleaved from the solid support by a TFA cocktail containing TES and H_2O (95/3/2) as scavengers. Finally, the peptides were purified by RP-HPLC and characterized by analytical HPLC and mass spectrometry. The final products were obtained with >95% purity (for more detail see Supporting Information).

Pharmacological assays

Membrane fragments from BHK-21 and CHO cell lines expressing the human Y1 and Y2 receptor subtypes, respectively, and HEK293 cell line expressing either the human Y4 or Y5 receptor subtypes were used. ^{125}I-PYY was used as radioligand for competition at the Y1, Y2 and Y5 receptors, and ^{125}I-PP was used as radioligand for competition at the Y4 receptor. The signal transduction assay for the human Y1, Y2 and Y4 receptor subtypes utilizes HEK293 cells expressing a biosensor developed around a modified rat olfactory cyclic nucleotide gated (CNG) calcium channel engineered to be cAMP selective and thus function as a cAMP responsive biosensor that signals through calcium. In the human Y5 receptor IPOne assay a HEK293 cell line stably expressing both the human Y5 receptor and the chimeric G-protein Gqi5 was used where Gqi5 ensures Gq signaling of the Gi coupled Y5 receptor.

NMR

For NMR structural studies a 1 mM solution of the peptide in 20 mM MES buffer, pH 5.5, in presence of 300 mM d38-DPC (Cambridge Isotopes) was used. All spectra were recorded at 37 °C on a Bruker 700 MHz Avance spectrometer equipped with a cryoprobe. For assignment purposes a 40 ms [^{1}H,^{1}H]-TOCSY and a 100 ms zero-quantum suppressed NOESY were measured and evaluated.[25] All spectra were assigned using the program XEASY.[26] Resonance assignments were largely following the sequential

resonance assignment procedure.[27] Assignment was largely facilitated by knowledge of proton chemical shifts of PYY1-36, for which resonances of the *C*-terminal α-helix were characterized by very similar resonance frequencies. All proton frequencies were deposited in the BMRB database under accession code 17291. NOEs were converted into upper-distance restraints, and used in restrained MD calculations using the program DYANA.[28] Out of 100 computed structures the twenty lowest energy conformers were selected to represent the NMR ensemble. The latter was further refined in explicit water using the program AMBER.[29] RMSD calculations and preparation of figures was accomplished using the program MOLMOL.[30] For further information on statistics of restraints, energy of computed conformers, Ramachandran statistics etc. see the Supporting information. The coordinates are deposited in the pdb database under accession code 2I60.

Membrane binding assay

Brain lipid vesicles were formed by standard rehydration of Folch bovine brain extracts (fraction 1, Sigma-Aldrich), supplemented with 0.5 mol % 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (DOPE-Biot.; Avanti Polar Lipids, Alabama, USA) and 0.5 mol % 3,3'-dioctadecyl-oxacarbocyanine perchlorate (DiO) (Invitrogen, Denmark) for surface immobilization and visualization respectively, according to Bhatia *et al.*[31] Briefly, unilamellar vesicles were prepared by first drying down lipids to a thin film, then rehydrating in sorbitol (equimolar to experiment buffer) overnight at 37 °C and finally the solution was subjected to 5 cycles of freeze/thaw and then extrusion (LipexTM Extruder, Northern Lipids, Burnaby, Canada,) through a poly-carbonate filter with a pore size of 0.8 μm (Millipore, Denmark).

Cleaned glass surfaces were functionalized with BSA-biotin, passivated with BSA and activated with Streptavidin in order to tether the brain lipid vesicles through biotin-streptavidin coupling as previously described.[31] Binding of peptide to tethered vesicles was performed in TBS (10 mM TRIS, 95 mM NaCl, pH 7.4). All micrographs were acquired on a Leica TCS SP5 Confocal Fluorescence Microscope, with an AOBS/AOTF system allowing tuneable wavelength detection intervals. The objective used was an oil immersion HCX PL APO with x100 magnification (NA 1.4). Vesicle fluorescence intensity were detected at 495–600 nm (exc. at 488 nm) while peptide-Atto655 (peptide **20** and **21**) fluorescence was detected at 640-780 nm (exc. at 633 nm) sequentially to avoid cross excitation and FRET. The micrographs were analyzed using a customized routine in Igor Pro 5.03v where threesholding, minimum particle area and ellipticity were used as tools to faithfully track particles and convert integrated intensity on the vesicle channel to vesicle diameter and intensity on the protein channel to protein density.[31]

Results and discussion

Design studies

Based on this hypothesis we first analyzed the membrane binding of PYY3-36. Following this, we tested whether mutating the 'address' segment of PYY3-36 could help to increase or modulate binding affinity and receptor signaling. Here we studied PYY3-36 and introduced new analogs with a novel architecture by appending an additional amphipathic α-helix to the existing α-helix in PYY3-36, via the relatively rigid natural turn segment (Figure 1).

Native human PYY3-36 displays high potency towards the Y2 receptor subtype but is only moderately selective as it also activates the Y1 and Y5 receptors.[32] Further *N*-terminal truncation to, for example, PYY13-36 (peptide **1**, Table 1 and 2) reduces the affinity towards the anorectic Y2 receptor significantly.[33] Our strategy was to introduce a second amphipathic α-helix at the *N*-terminus of PYY13-36 which contains the natural turn motif, followed by the α-helix ('address') and the receptor binding pentapeptide ('message'), This would provide a helix-turn-helix sequence (Figure 1). The new, appended α-helix had to be amphipathic and bind to the outer leaflet of the membrane. For this, we chose a *de novo* designed peptide sequence with a general heptad repeat structure (which previously has been used in artificial proteins)[34-36] as a general platform for further modifications. First, we studied which modifications were compatible with Y2 receptor binding, especially which could lead to a recovery of the high Y2 receptor affinity of PYY3-36, while improving the membrane binding. The first peptide in this series, peptide **2**, had a significantly lower Y2 receptor affinity than native PYY3-36 and even the truncated peptide **1** (Table 1 and 2). This indicated that the particular structure incorporated to increase membrane affinity had to be balanced with the need for maintaining receptor docking. A modest 3-fold improvement in Y2 receptor affinity, relative to peptide **2**, was obtained by inverting the chirality of the Pro in the linker region, peptide **6**. Also substituting Tyr for Ala, peptide **5**, and Glu for Ser, peptide **12**, gave moderately improved Y2 receptor binding, in a manner which interestingly seemed to correlate with the pI. While other modifications, such as Leu to Phe or Trp mutations, or extension of the appended α-helix lowered the Y2 receptor affinity. However, only minor changes in Y2 receptor affinity and potency were observed (peptides **3**-**14**, Table 1 and 2). In contrast, extending the linker segment between the two α-helices with the GEDA sequence from PYY3-36 (peptide **15**) improved both binding (7-fold) and activity (3-fold) at the Y2 receptor compared to peptide **2**. Interestingly, the extended linker segment also resulted in a reduced Y1 and Y4 receptor binding affinity.

To further improve the activity of the linear 2-helix analogs, the artificial *N*-terminal α-helix of peptide **2** was exchanged by other heptad repeat sequences[37-39] (peptides **16**-**19**). The *de novo* α-helix of

peptides **16**, **17** and **19** are more hydrophilic compared to the α-helices of peptides **2**-**15**, and peptide **18** is more hydrophobic. In peptides **16** and **17** residues at position *c* and *g* of the heptad repeat segment were swapped (LKELERK in peptide **16** vs. LKKLERE in peptide **17**). Peptides **16** and **17** both displayed a 2-3 fold increased binding at the Y1 receptor, slightly less for peptide **16**, and 10-fold increased binding to the Y2 and Y5 receptors compared to peptide **2** (Table 1). The signal transduction assay of peptide **16** and **17** confirmed the receptor selectivity obtained from the binding experiments (Table 2). The more hydrophobic peptide **18** showed as low binding to all Y receptors and low activity as peptide **2**. Altering the heptad repeat α-helix such that the positively charged Lys residues were clustered in the center of the heptad repeat (peptide **19**) resulted in very high Y2 receptor affinity and potency but, unfortunately, only in minor decreases in binding affinity to the Y1 and Y5 receptor compared to PYY3-36 (Table 1 and 2). Interestingly, although peptide **19** had an Y1 receptor affinity similar to PYY3-36, the signal transduction assay showed a significantly lowered potency at the Y1 receptor, thus peptide **19** was a nanomolar Y2 receptor agonist with improved Y2/Y1 receptor selectivity. However, as peptide **19** retained an unfavorably high Y5 potency, peptide **17** was chosen for further studies.

To summarize our design studies, novel PYY3-36 analogs with high binding affinity and potency, in addition to good selectivity to the Y2 receptor were derived upon addition of a heptad repeat amphipathic α-helix to the truncated *C*-terminal α-helix of PYY. We focused on the natural pentapeptide SPEEL as the linker between the two α-helices. The distribution of charged or aromatic residues on the appended α-helix was crucial for achieving high affinity and selectivity. Generally, it seems that the more hydrophilic amphipathic heptad repeat sequences led to the highest Y2 receptor binding and potency. Interestingly, the most potent Y2 receptor agonists also activated the Y5 receptor indicating that ligand-receptor interactions are very similar between the Y2 and Y5 receptors. Thus, radically redesigned PYY3-36 variants with high potency and new properties were developed. Peptide **17** was chosen for further detailed biophysical studies because it presents the best compromise in terms of receptor affinity and receptor subtype selectivity.

Structural studies

In order to understand possible structural implications of the modifications several biophysical methods were used to characterize the peptides. CD spectra of PYY3-36 showed a degree of α-helicity of approx. 28%. The novel Y2 agonists displayed α-helicities in solution of 25-52% (Table 2). CD indicated that the novel 2-helix PYY analogs possess medium to high α-helicity. A correlation between α-helicity and activity was, however, not observed, except as shown for peptide **14**, where a low α-helicity was associated with an abolishment of activity. CD spectroscopy of both PYY3-36 and peptide **17**, both in

solution and in the association with lipid membranes (from standard rehydration of Folch bovine extracts) revealed a high content of α-helicity (Supporting Information). This is especially the case with dodecylphosphocholine (DPC) micelles, a detergent that contains the choline headgroup present in the outer leaflet of mammalian membranes.[40]

Next we investigated the structure of peptide **17** in more detail by solution NMR. To characterize the membrane-bound state of peptide **17** we determined the structure of the peptide in presence of DPC micelles. The structure was determined based on upper-limits derived from nuclear Overhauser effects (NOEs) (for further details on the structure calculation see Table 3 as well as the Supporting Information). An expansion of the amide region displaying NOEs involving sequential amide protons as well as a single conformer of peptide **17** determined by NMR spectroscopy are depicted in Figure 2. The structure reveals the presence of two amphipathic α-helices encompassing residues 3-13 and 18-33. Both α-helices are well defined, and the individual superposition of backbone atoms of residues 3 to 13 or 18 to 33 yields RMSD values of 0.52 or 0.48 Å, respectively, and 1.92 Å when backbone atoms of both α-helices are superimposed. The *C*-terminal pentapeptide at the α-helix terminus of this 2-helix PYY variant, peptide **17**, is slightly less well defined than in PYY, as reflected in RMSDs of 1.1 vs. 0.76 Å, respectively, for backbone atoms of the *C*-terminal α-helix. However, the presence of both sequential as well as medium-range NOEs at the end indicates that differences between PYY and peptide **17** are small (Supporting Information). Interestingly, the orientation of the α-helices with respect to each other is also defined, supported by a number of medium-range NOEs in the hinge region.

The two α-helices are very well defined with apolar and polar residues clustered on opposite faces of the α-helices. In order to establish the topology of membrane-peptide association we measured distance-dependent paramagnetic relaxation enhancement in the presence of the micelle-integrating spin label 5-doxylstearate.[41] Attenuation of signals in the [^{15}N,^{1}H]-HSQC spectrum was measured after addition of the micelle-integrating spin-label. Strongest attenuation of signal, due to vicinities of the amide moieties to the membrane-water interfacial region, was observed for non-polar or aromatic residues, with the most notable exception being a strong reduction in signal intensity for Lys-14 (Supporting Information). The data reveal that the peptide associates with the micelle surface such that the apolar residues point towards the interface and the charged residues to the opposite (aqueous) side.

Membrane binding

PYY3-36 was *N*-terminally labeled with the fluorophor ATTO655 (for structure see Supporting Information) to provide peptide **20** for membrane affinity studies. A fluorescently labeled version of

peptide **17** was constructed by incorporation of an *N*-terminal Cys, which was S-alkylated with maleimide functionalized ATTO655 to provide peptide **21** (Supporting Information). We have previously reported point mutations in peptides labelled with ATTO dyes, with supports that the membrane binding of amphipathic peptides is dominated by their sequence and not the dye.[42-43] The membrane binding of fluorescently labeled **20** (ATTO655-PYY3-36) and **21** (ATTO655-peptide **17**) were also studied on fluorescently labeled single small unilamellar vesicles (Folch fractions from bovine brain, see methods) using confocal fluorescence microscopy (Figure 3).[44] Liposomes were immobilized by tethering on passivated glass surfaces through streptavidin-biotin linkages,[45] and were isolated at the single particle level simply by diluting their surface density several times below the optical resolution.[31,43-46] The exact diameter of each single vesicle (±5 nm) as well as the amount of labeled peptide bound on each liposome were calculated by integrating the respective fluorescence intensity signals and calibrating them using a procedure described in detail by Hatzakis *et al*.[43] (Figure 3). The ratio between the number of lipids and amount of bound peptides provided the density of peptide on the single vesicles.[43]

Titration of peptide **21** at concentrations in the range from 10 nM to 200 nM to vesicles allowed recording the binding curve for the peptide-membrane interaction (Figure 3) and to extract the Kd from the fit as 4 nM. The binding of peptide **21** to the membrane reached a saturation density at ~50 nM (Figure 3). PYY3-36, in contrast, did not reach saturation even at 600 nM, at which concentration the membranes began to deform. Consequently, the Kd for PYY3-36 towards the membrane could not be determined. Nevertheless, the fact that peptide **21** reached saturation at >12 fold lower concentration illustrates the prominent increase in membrane recruitment for peptide **21** (and **17**) compared to PYY3-36. In comparison, PYY1-36 has been reported to bind to DMPC and a 4:1 mixture of DMPC/DMPG vesicles in the micro molar range (determined by surface plasmon resonance experiments),[47] however, to our knowledge, no studies have been performed on PYY3-36.

Discussion

We have studied the ability of PYY3-36 to bind to membranes and designed a series of novel Y2 receptor ligands exploring the fact that the NPY family of peptides associates with the plasma membrane. We started from pharmacological screening and then proceeded to biophysical characterization of a single peptide. In order to increase membrane affinity of the peptides an amphipathic α-helix was appended at the *N*-terminal end of the original α-helix of PYY, i.e. the *N*-terminal part was exchanged for an artificial α-helix. The resulting analogs of PYY3-36 containing the more hydrophilic *de novo* designed helices lead to the most potent Y2 receptor ligands. The novel 2-helix PYY3-36 analogs displayed nanomolar Y2 receptor potency, which in some cases was accompanied by an increase in Y2 receptor selectivity (Y2 over Y1 or Y4). Interestingly, these analogs are similarly potent at the Y5 receptor. The data indicate that

ligand-receptor interactions of Y2 and Y5 receptors are very similar, and supports previous work by Beck-Sickinger and co-workers using a complementary mutagenesis approach with NPY as the ligand.[48]

In order to further understand the membrane binding properties of the redesigned peptides, peptide **17** which displayed an interesting pharmacological profile was chosen for biophysical investigations. The NMR structure of peptide **17** in the presence of a membrane mimetic displayed two well-defined α-helices encompassing residues 3-13 and 18-33. Polar and apolar residues were clustered on different faces of the α-helices (Figure 2). The presence of a number of NOEs between residues of the *N*- and *C*-terminal helix proved that the two α-helices were not flexibly linked but rather adopted an L-shaped relative orientation. NMR showed only minor conformational differences in the *C*-terminal receptor-binding pentapeptide between peptide **17** and PYY3-36, likely responsible for the similarity in the Y2 receptor binding data.

Previous studies have demonstrated that PYY1-36 and PYY3-36 share a common membrane-binding mode.[20-21] Moreover, the pharmacological assays revealed that some of the PYY3-36 analogs described here, which were designed to more tightly associate with the membrane, maintained high Y2 receptor affinity. The novel nanoscale surface-based assay provides unique information about the membrane binding capacity of the peptide. Since the two α-helices in peptide **17** are not flexibly linked but rather form an L-shaped molecule, the architecture of the amphipathic nature of the peptide is well suited for binding to membranes. We speculate that these two features help in the recruitment to the membrane, and in the accumulation of the peptide in membrane compartments. In that respect it is of interest that it has been previously hypothesized that the amphipathic 8^{th} helix of the rhodopsin-like family of GPCRs can act as a sensor for lipid composition or membrane curvature.[49] Moreover, the GPCRs may introduce lipid packing defects that can induce membrane bending,[43] resulting in recruitment of ligands like PYY towards the receptors.[50-51] In that context it is important to note that GPCRs are not uniformly distributed in the plasma membrane but are rather enriched in discrete areas of the plasma membrane such as lipid rafts that display distinct lipid and protein composition and membrane thickness.[52] Varying the membrane affinity may constitute a tool for creating peptides with selectivity for receptor populations in specific membrane environments that could affect the pharmacological profile of the peptides in a tissue-dependent manner.

Association of peptides with membranes is not expected to influence Y receptor subtype selectivity as such. Improved Y receptor subtype selectivity thus is rather due to direct interactions with the receptor. Also, a peptide with improved membrane binding would still need to correctly dock into the receptor to exhibit the desired effect. Thus, the method for increasing membrane binding may have to be optimized

for a particular peptide to not interfere with receptor docking. Although peptide **17** bound to model membranes with higher affinity compared to PYY3-36, this apparently was not directly translated into higher affinity at any of the other Y receptors under the conditions of the present study. It is likely that the addition of the heptad repeat sequences can affect receptor affinity negatively. Indeed, the first 2-helix variant, peptide **2**, had a 65-fold reduced Y2 receptor affinity compared to PYY3-36. It is noteworthy that compared to peptide **2** the affinity of peptide **17** was increased for the Y1, Y2, and Y5 receptors (10-fold in case of the Y2 receptor). Thus, a new strategy for modulating and optimizing the properties of peptide hormones has been established.

Conclusion

In conclusion, we herein present a radical redesign of the pharmacologically important peptide hormone PYY3-36 to improve membrane binding. Appending an additional amphipathic helix to form a novel 2-helix variant increased the membrane-binding affinity over the native PYY3-36. The new 2-helix variant retained a high binding affinity for the Y2 receptor as well as the activity. NMR studies revealed that when bound to DPC micelles both helices were fully formed and adopted a rigid L-shape. The membrane binding was related to the structure of the peptide, and in particular to the amphipathic nature of the two helices and their relative orientation. We have herein demonstrated that when properly choosing both the appended α-helix and the linker segment that connects the two helices both affinity and potency can be retained. These findings contribute to the hypothesis that membrane binding of amphipathic peptide hormones may play an important role in the binding to their membrane-bound receptors. Finally, the new concept demonstrated here for the redesign of the architecture of a biopharmaceutically important peptide can likely be extended to other important peptides for the development of potential drug candidates.

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Figures and Tables

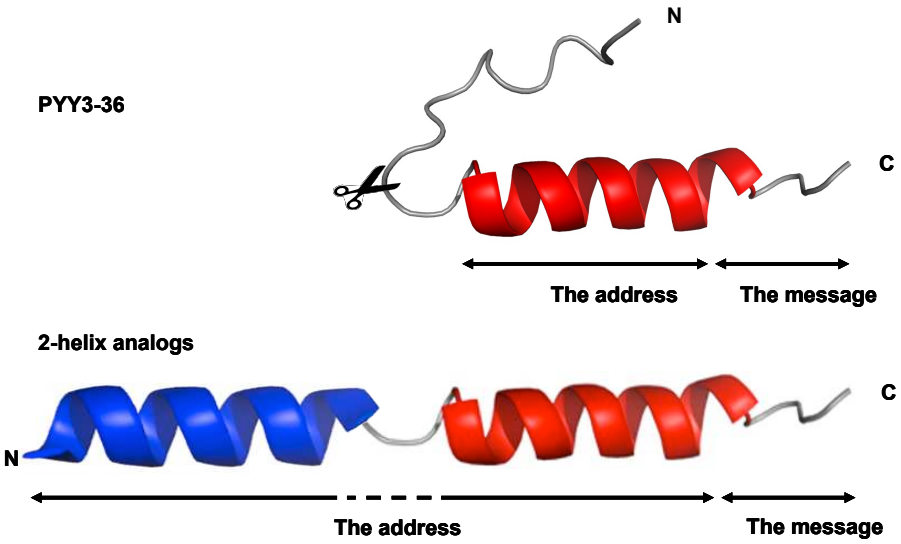


Figure 1. The peptide design concept. The *N*-terminal half of PYY is replaced by an amphipathic α-helix. The native PYY α-helix is depicted in red and the appended α-helix in blue.

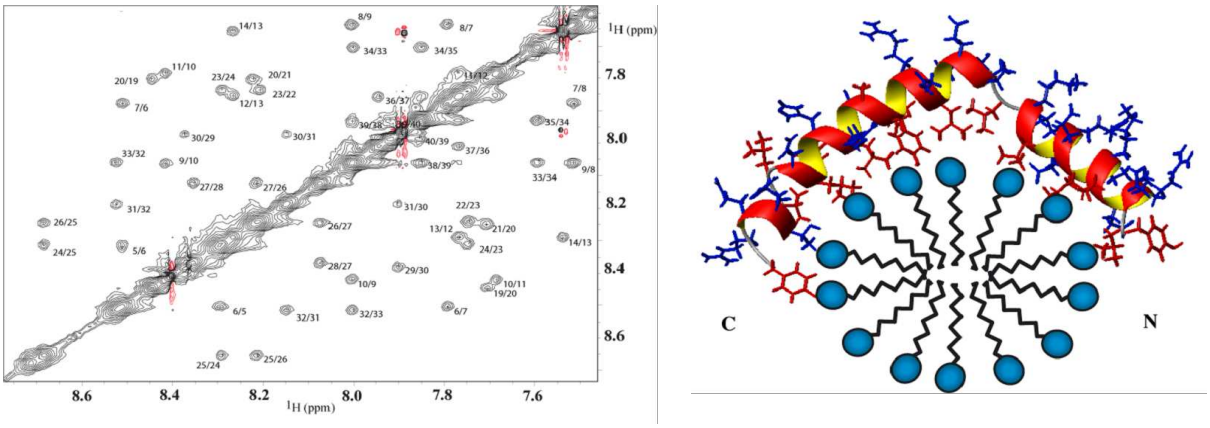


Figure 2. Left: Expansion of the amide proton region of the NOESY spectrum of peptide **17**. Cross peaks corresponding to sequential contacts are annotated. Right: Single conformer of peptide **17** as determined by solution NMR spectroscopy. Hydrophobic and polar side-chains are depicted in red and blue, respectively. The position of the micelle lipids is schematically shown.

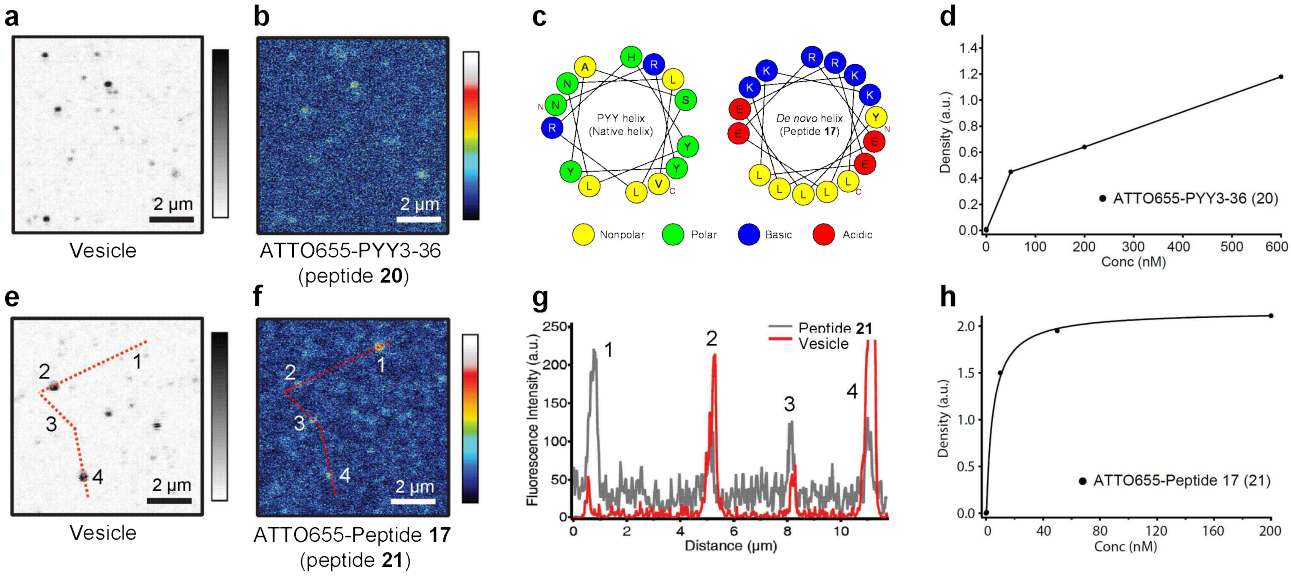


Figure 3. PYY3-36 and peptide **17** bind to brain lipid membranes. Single brain lipid vesicles of different size were immobilized on a glass surface using streptavidin-biotin linkage and imaged with confocal microscopy. Micrographs of vesicles (exc. 488 nm) (a and e) and labelled peptide (exc. 633 nm) respectively ATTO655-PYY3-36 (peptide **20**) and ATTO655-peptide **17** (peptide **21**) (b and f, respectively). The fluorescence intensities extracted from the images are used to quantitatively calculate liposome size and the density of bound peptide. Scale bar is 2 µm and applies to all micrographs. To guide the eye, data points have been simply connected by straight lines (e and f). Intensity profiles of cross section indicated by red line in e and f, illustrating the binding of peptide (grey) to vesicle membrane (red) by colocalized signal (g). Projection of native PYY3-36 helix and *de novo* α-helix in a helical wheel, demonstrating the increased amphipathicity (c). Binding of ATTO655-PYY3-36 (peptide **20**) at different concentrations to vesicle membranes with a diameter of 75 (±5) nm (n=1009) (d). Saturation was not reached even at 600 nM, hence a Kd value for PYY3-36 could not be calculated. Binding curve of ATTO655-peptide **17** (peptide **21**) to vesicle membranes with a diameter of 75 (±5) nm (n=715) (h). The Kd of peptide **17** was calculated from the fit to be 4 nM.

Table 1. Y receptor binding affinity of native PYY3-36 and the novel 2-helix PYY analogs.

		Binding affinity assay IC_{50} [nM]			
Peptide	*N*-terminal[a]	Y1R	Y2R	Y4R	Y5R
PYY3-36	H-IKPEAPGEDASPEEL-X	32 ± 19	0.54 ± 0.15	255 ± 29	3.55 ± 1.86
1	H-SPEEL-X	110 ± 59	6.63 ± 2.39	>1000	47.13 ± 14.09
2	H-YEELLKKLEELLKKASPEEL-X	655 ± 234	35.97 ± 12.35	>1000	41.63 ± 15.37
3	Ac-YEELLKKLEELLKKASPEEL-X	>1000	67.00 ± 26.31	>1000	99.43 ± 28.51
4	H-IKPEAPGYEELLKKLEELLKKASPEEL-X	>1000	43.83 ± 14.16	>1000	57.43 ± 14.24
5	H-AEELLKKLEELLKKASPEEL-X	661 ± 203	14.80 ± 5.54	>1000	39.87 ± 35.91
6	H-YEELLKKLEELLKKASpEEL-X	324 ± 98	12.17 ± 1.21	>1000	7.74 ± 1.48
7	H-YEELLKKLEELLKKASPeEL-X	793 ± 169	22.63 ± 9.32	>1000	22.83 ± 5.69
8	H-YEELLKKLEELLKKASPEeL-X	>1000	42.33 ± 15.14	>1000	49.37 ± 20.71
9	H-YEELLKKLEELLKKASpeEL-X	528 ± 243	19.77 ± 4.13	>1000	13.84 ± 4.32
10	H-YEEFFKKLEELFKKASPEEL-X	>1000	84.80 ± 22.99	>1000	42.43 ± 10.28
11	H-YEEWWKKLEELWKKASPEEL-X	>1000	106.50 ± 18.62	>1000	39.27 ± 15.87
12	H-YESLLKKLSELLKKASPEEL-X	400 ± 141	16.43 ± 4.69	>1000	34.67 ± 13.05
13	H-YEELLKSLEELLKSASPEEL-X	>1000	60.57 ± 28.51	>1000	150.57 ± 66.71
14	H-YEELLKYLEELLKYASPEEL-X	>1000	279.50 ± 14.85	>1000	387.00 ± 266.91
15	H-YEELLKKLEELLKKAGEDASPEEL-X	179 ± 44	5.29 ± 2.20	>1000	7.10 ± 1.22
16	H-YLERKLKELERKLKELSPEEL-X	260 ± 109	2.72 ± 1.90	>1000	9.36 ± 1.56
17	H-YLERELKKLERELKKLSPEEL-X	154 ± 72	3.22 ± 1.55	>1000	10.45 ± 1.58
18	H-YLKALKEALKALKEALKSPEEL-X	338 ± 110	27.00 ± 10.50	664 ± 276	35.30 ± 5.63
19	H-NLEELKKKLEELKGSPEEL-X	36 ± 24	1.84 ± 0.62	532 ± 354	5.35 ± 0.84

a) X = NRYYASLRHYLNLVTRQRY-NH_2 (PYY18-36)

Table 2. Y receptor potency of native PYY3-36 and the novel 2-helix PYY and their degree of α-helicity.

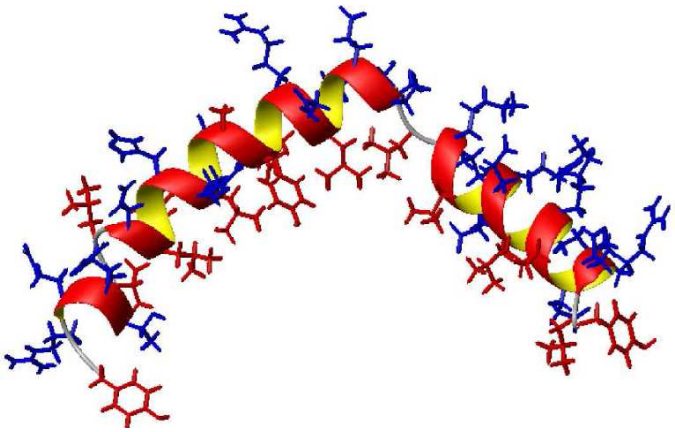
		Signal transduction assay EC_{50} [nM]				α-helicity[b]
Peptide	*N*-terminal[a]	Y1R	Y2R	Y4R	Y5R	%
PYY3-36	H-IKPEAPGEDASPEEL-X	14.00 ± 5.29	1.14 ± 0.27	>100	3.54 ± 0.06	26
1	H-SPEEL-X	>100	9.4 ± 1.4	>100	39.67 ± 8.39	32
2	H-YEELLKKLEELLKKASPEEL-X	>100	37 ± 5	>100	28.00 ± 1.73	45
3	Ac-YEELLKKLEELLKKASPEEL-X	>100	51 ± 7	>100	40.33 ± 3.79	40
4	H-IKPEAPGYEELLKKLEELLKKASPEEL-X	>100	>100	>100	57.67 ± 7.02	40
5	H-AEELLKKLEELLKKASPEEL-X	>100	55 ± 12	>100	25.00 ± 1.00	-
6	H-YEELLKKLEELLKKASpEEL-X	>100	26 ± 7	>100	9.47 ± 2.55	31
7	H-YEELLKKLEELLKKASPeEL-X	>100	74 ± 8	>100	21.33 ± 6.66	27
8	H-YEELLKKLEELLKKASPEeL-X	>100	89 ± 16	>100	32.67 ± 5.69	25
9	H-YEELLKKLEELLKKASpeEL-X	>100	39 ± 1	>100	9.14 ± 3.85	16
10	H-YEEFFKKLEELFKKASPEEL-X	>100	>100	>100	28.33 ± 3.06	42
11	H-YEEWWKKLEELWKKASPEEL-X	>100	>100	>100	32.33 ± 2.31	50
12	H-YESLLKKLSELLKKASPEEL-X	>100	59 ± 7	>100	35.00 ± 3.61	28
13	H-YEELLKSLEELLKSASPEEL-X	>100	32 ± 6	>100	40.00 ± 6.08	44
14	H-YEELLKYLEELLKYASPEEL-X	>100	>100	>100	68.00 ± 15.13	8
15	H-YEELLKKLEELLKKAGEDASPEEL-X	>100	13 ± 2	>100	4.24 ± 1.20	16
16	H-YLERKLKELERKLKELSPEEL-X	>100	19 ± 3	>100	15.33 ± 2.52	37
17	H-YLERELKKLERELKKLSPEEL-X	>100	15 ± 3	>100	11.33 ± 1.53	39
18	H-YLKALKEALKALKEALKSPEEL-X	>100	>100	>100	61.33 ± 23.18	-
19	H-NLEELKKKLEELKGSPEEL-X	>100	5.4 ± 0.5	>100	4.60 ± 0.53	26

a) X = NRYYASLRHYLNLVTRQRY-NH_2 (PYY18-36)

b) Determined by CD spectroscopy

Table 3. Structural statistics for the peptide **17**.	
NMR distance restraints:	
Total NOE	429
Short-range, $\|i - j\| \leq 1$	304
Medium-range, $1 < \|i - j\| < 5$	124
Long-range, $\|i - j\| \geq 5$	1
Maximal distance restraint violation	0.25 Å
AMBER energies:	
Total (mean and s.d. of 20 conformers)	−1287 ± 66 kcal/mol
van der Waals	−45 ± 13 kcal/mol
RMS deviations from idealized geometry:	
Bond lengths	0.0259 ± 0.0011 Å
Bond angles	2.00 ± 0.04°
Ramachandran plot statistics:	
Residues in most favored regions	78%
Residues in additionally allowed regions	20%
Residues in generously allowed regions	1%
Residues in disallowed regions	1%
RMS deviations from the mean coordinates:	
N, C^{α}, C' of residues 3–13, 18-33	1.92 Å
N, C^{α}, C' of residues 3–13	0.52 Å
N, C^{α}, C' of residues 18–33	0.48 Å

Table of Contents



A radically redesigned 2-helix PYY3-36 analog shows improved membrane binding yet has a high receptor affinity